The protective role of uteroglobin through the modulation of tissue transglutaminase in the experimental crescentic glomerulonephritis

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Abstract

Background and methods. Tissue transglutaminase (tTG) may induce pro-inflammatory cytokines and produce irreversible end-products, thus promoting renal scarring. It has recently been confirmed that the crescent formation in murine experimental crescentic glomerulonephritis (ecGN) has been inhibited by the administration of recombinant uteroglobin (rUG). However, the ability of UG on tTG modulation has not been thoroughly assessed. In this study, we investigated the feasible protective role of UG in murine ecGN through the modulation of tTG and TGF-β1 expressions. ecGN was induced by the administration of anti-GBM Ab into C57BL/6 mice.

Results. Both proteinuria and BUN levels were distinctively lower in rUG-treated mice compared to those of disease control mice. Glomerular injuries such as mesangial proliferation, matrix production and crescent formation were lessened with the rUG treatment, and these findings were parallel with the attenuated expression of tTG and TGF-β1. tTG and TGF-β1 were expressed mainly on mesangial areas by the induction of ecGN and rUG treatment markedly attenuated the expressions of these proteins in glomeruli without spatial changes. With the addition of LPS to mesangial cells, the expressions of TGF-β1 were up-regulated, whilst the addition of cysteamine, tTG inhibitor, attenuated the expression of tTG and TGF-β1 as well as the cellular proliferation which was further induced by LPS.

Conclusion. We demonstrate for the first time that rUG is able to attenuate the renal injury through the modulation of expressions of tTG and TGF-β1 in ecGN and further suggest a wide range of feasible molecular targets to reduce the severity of human glomerulonephritis.

Keywords: experimental crescentic glomerulonephritis; tissue transglutaminase; uteroglobin

Introduction

In spite of the growing prevalence of diabetic nephropathy, glomerulonephritis (GN) remains a major cause of chronic kidney disease and end-stage renal disease requiring dialysis and transplantation in Korea as well as worldwide [1,2]. Glomerular crescent formation, which serves as a hallmark of severe renal injury, predicts the poor prognosis of GN and is characterized by the accumulation of T cells, macrophages and fibrin in glomeruli [3,4]. Anti-glomerular basement membrane Ab (anti-GBM Ab)-induced GN is a well-characterized model of crescentic GN and appears to be the most similar counterpart of human disease [3,5]. The underlying mechanism of crescent formation is complicated which further requires extensive exploration of CD4 T cells, inflammatory cytokines and chemokines [6,7].

TGF-β1 is a representative profibrotic cytokine and plays a key role in the progression of chronic kidney disease and crescentic GN. Blockade of the downstream of TGF-β1/Smad signalling is capable of inhibiting both renal fibrosis and inflammation [8,9]. In addition, with the blocking of TGF-β1 via receptor modulation, glomerular injury and interstitial fibrosis were ameliorated after anti-GBM Ab administration [10,11]. TGF-β1 is susceptible to inhibit matrix metalloproteinase and plasmin synthesis as well as inducing their inhibitors [12]. Recently tissue transglutaminase (tTG), extracellular matrix modulating enzyme, has been reported to be linked to many actions of TGF-β1 in the scarred kidney [13]. tTG is able to irreversibly cross-link through ε-γ-glutamyl-lysine isopeptide bonds between lysine and glutamine residues, thereby facilitating inappropriate deposition of ECM proteins [14]. Uteroglobin (Ug) is a steroid-dependent, immunomodulatory and cytokine-like protein [15,16]. It appears to
function as an anti-inflammatory agent in both of respiratory and urogenital tracts [17]. Previously, we have reported that the administration of exogenous UG prevented the experimental crescentic GN (ecGN) based on the inhibition of deposition of Ab and complement-3 on the glomeruli [5]. Substantially, it has been recognized by several experiments that UG is an excellent substrate of tTG [16,18].

In this study, we investigated the possible protective role of UG in a murine model of ecGN through the modulation of TGF-β1 and tTG expressions.

**Materials and methods**

**Production of recombinant human uterogoblin**

Human lung cancer cell line (NCI-H322) (ATCC, CRL-5806) was used for cloning the human UG. To obtain the whole segment of mature UG, the reverse transcription-polymerase chain reaction (RT-PCR) was conducted using CC10-1 (sense primer 5′-CCAGACTCAGAGACGGAAA-3′) and CC10-2 (antisense primer 5′-CATATGAAACTCGCTGTGACC-3′). Nested PCR was performed after first round PCR using CC10-1 (sense primer 5′-CTCGAGGCTTACATACGAGCT-3′) and CC10-4 (antisense primer 5′-GACTCAACATGGCAGGACG-3′). PCR product was cloned into pET32a plasmid (Novagen, Madison, WI, USA). Recombinant uterogoblin (rUG) was produced from transformed E. coli BL21. His-tagged rUG was purified by Ni2+-affinity chromatography (Novagen). Centrifonic (Millingpo, Bedford, MA, USA) was used to concentrate the recombinant protein after dialysis, and the Bradford assay was used to quantify the protein (Bio-Rad Laboratories, Hercules, CA, USA). rUG was confirmed by western blot where the polyclonal rabbit anti-human UG antibody (urine protein 1) (Dako- Cytomation, Glostrup, Denmark) was used as a primary antibody (data not shown).

**Mesangial cell proliferation assay**

Murine mesangial cells (1 × 10^5/well, SV40 MES 13) were plated into a 96-well plate. Cells were maintained in the DME:F12 (3:1) medium and were supplemented with both 14 mmol/l HEPES and 5% fetal bovine serum. After 24 h, the culture media were changed to media containing 0.5% fetal bovine serum (FBS). The cells were then washed with PBS. The culture media were changed for one containing 1% FBS, 1% FBS + LPS (2 µg/ml) and 1% FBS + rUG (10, 25 and 50 µg/ml). Cells were then washed in PBS and fixed in 4% paraformaldehyde in PBS. After blocking with 5% BSA, cells were incubated with a rabbit anti-transglutaminase-2 antibody (NeoMarkers, Union City, CA, USA) for 16 h at 4°C. A second layer of the Alexa Fluor® 488-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR, USA) in mesangial cells was assessed using confocal laser-scanning microscopy (Carl Zeiss, Jena, Germany) under ×200 magnification. Nuclei were visualized using 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes). Renal tissues were harvested from mice 7 days after disease induction. For immunofluorescence study, cryostat sections of snap-frozen kidney were cut into 4 µm slices. The sections were stained for 1 h at room temperature with mouse anti-transglutaminase-2 Ab (NeoMarkers). A second layer of the Alexa Fluor® 555-conjugated goat anti-mouse antibody (Molecular Probes) was applied and incubated at room temperature for 1 h. The sections were washed and then incubated for 5 min with DAPI (Molecular Probes). Primary antibodies were omitted from sections used as negative controls. Sections were blindly and randomly evaluated.

**Confocal microscopic examination**

For detection of extracellular tTG, monolayers of murine mesangial cells (1 × 10^5/well, SV40 MES 13) in eight-well plastic chamber slides were cultured with each well in the growth medium [DME:F12 (3:1) medium, 14 mmol/l HEPES, 5% FBS]. After 24 h, the supernatant was withdrawn from each well and starved to 0.5% FBS. Cells were washed with PBS two times after 24 h, and the culture media were changed for one containing 1% FBS, 1% FBS + LPS (2 µg/ml) and 1% FBS + rUG (10, 25 and 50 µg/ml). Cells were then washed in PBS and fixed in 4% paraformaldehyde in PBS. After blocking with 5% BSA, cells were incubated with a rabbit anti-transglutaminase-2 antibody (NeoMarkers, Union City, CA, USA) for 16 h at 4°C. A second layer of the Alexa Fluor® 488-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR, USA) in mesangial cells was assessed using confocal laser-scanning microscopy (Carl Zeiss, Jena, Germany) under ×200 magnification. Nuclei were visualized using 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes). Renal tissues were harvested from mice 7 days after disease induction. For immunofluorescence study, cryostat sections of snap-frozen kidney were cut into 4 µm slices. The sections were stained for 1 h at room temperature with mouse anti-transglutaminase-2 Ab (NeoMarkers). A second layer of the Alexa Fluor® 555-conjugated goat anti-mouse antibody (Molecular Probes) was applied and incubated at room temperature for 1 h. The sections were washed and then incubated for 5 min with DAPI (Molecular Probes). Primary antibodies were omitted from sections used as negative controls. Sections were blindly and randomly evaluated.

**Induction of experimental crescentic glomerulonephritis (ecGN)**

Anti-GBM Ab was prepared from the serum of a rabbit immunized against homogenized murine renal cortex in complete Freund’s adjuvant (Sigma, St. Louis, MO, USA) as prescribed previously [5]. ecGN was initiated by the intravenous injection of 3 mg of rabbit anti-GBM Ab into an 8- to 10-week-old male C57BL/6. Disease control mice (n = 8) received three doses of 300 µl of PBS after anti-GBM Ab injection. In treated mice (n = 8), rUG (500 µg in 300 µl of phosphate-buffered saline (PBS)/mouse for 3 days) was delivered 1 h after anti-GBM Ab injection via a tail vein. Normal control mice (n = 8) received PBS instead of anti-GBM Ab and renal injury was studied after 7 and 14 days. Proteinuria and urine creatinine were measured by 24-h urine collections at 7 and 14 days. Urinary protein concentrations were determined by the Bradford method. Creatinine concentrations were measured using an autoanalyzer. Proteinuria was normalized by urine creatinine (urine protein/urine creatinine; mg/mg). Serum was collected via retro-orbital bleeding at the same time of evaluation. Serum blood urea nitrogen (BUN) levels were measured by the urease-ultraviolet method using Urea NB (Wako Chemical Industries Ltd, Osaka, Japan). All the mice were raised in specific pathogen free animal facility and the treatment protocol was reviewed by Seoul National University Hospital Institute Review Board.
Histological analysis

To assess their light microscopic appearance, 4-µm paraffin sections were used to evaluate renal scarring. Glomerular sclerosis (GS) (periodic acid-Schiff and PAS) and Masson's trichrome stain. For grading the induced GN, the numbers of glomeruli forming crescent were counted in a blinded fashion. A minimum of 50 glomeruli per mouse kidney were evaluated, and the mean value was used as representative for the mouse. Glomerular sections were assessed by standard semiquantitative analysis and expressed as the glomerular sclerosis index (GSI) [19]. The extent of GS was graded from 0 to 4 by a semiquantitative (score: 0, normal glomeruli; 1, mesangial thickening of <25% of the tuft; 2, moderate GS-mesangial proliferation and thickening up to 50%; 3, severe GS-obliteration of capillaries and diffuse sclerosis up to 75% and 4, complete capillary obliteration and thrombosis with global sclerosis up to 100%). The GSI was given to each microscopic 10 fields viewed at ×200 magnification on PAS. The mean glomerular cross-sectional area was determined in at least 50 glomerular sections. Interstitial fibrosis score (%) was given to each microscopic 10 fields viewed at ×200 magnification on blue with Masson's trichrome stain. The lesions were quantified by a colour image analyser (Image-Pro-Plus for Windows). The fibrotic area was digitized and subjected to colour threshold analysis. Scores from 10 non-overlapping fields per kidney were final average percentage positive stain. For immunohistochemistry study, paraffin embedded kidney was cut into 4-µm slices. For deparaffinization and hydration, xylene and ethanol were used. Endogenous streptavidin activity was blocked by 0.3% hydrogen peroxide (H₂O₂). To examine the expression of TGF-β1 and type IV collagen, deparaffinized sections were stained with rabbit anti-TGF-β1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-type IV collagen Ab (Abcam, Cambridge, UK). Antigen retrieval was carried out by heating paraffin embedded sections in 10% citrate buffer in a microwave oven, three times, each for 5 min. Streptavidin and 3, 3'-diaminobenzidine tetrahydrochloride (Sigma) were used for immunohistochemical detection. The glomerular anti-TGF-β1 and type IV collagen positive area to the glomerular tuft area were calculated by image analysis using Olympus optical. A minimum of 50 glomeruli were evaluated per mouse and the mean values are expressed as cell per glomerular cross-section (cells/gcs). Sections were then counterstained with Mayer's haematoxylin and examined by light microscopy.

Western blot analysis

Kidneys were harvested 7 days after inducing ecGN. Western immunoblot analysis of kidney was performed using the primary antibodies against anti-transglutaminase-2, TGF-β1 and β-actin to show specificity to their respective antigens. Briefly, kidney tissue was homogenized in PBS containing complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). The kidney homogenate was centrifuged at 12 000 × g for 30 min at 4°C, and protein concentration was determined on the supernatant by the Bradford method. Equal amounts (60 µg) of extracted protein were separated by 12% SDS-polyacrylamide gels (PAGE) and transferred onto Immobilon-FL 0.4 µM polyvinylidene difluoride membranes (Millipore). Tris-buffered saline containing 0.1% Tween-20 was used as washing buffer. Abs used were as follows: anti-transglutaminase 2 (Neomarkers) and TGF-β1 (Cell Signaling Technology, Danvers, MA, USA). As a loading control, the level of β-actin was evaluated using anti-β-actin antibody (Cell Signaling Technology). The secondary antibodies used were anti-rabbit (Cell Signaling Technology) and anti-mouse (Cell Signaling Technology) Abs, respectively. Detection of labelled proteins was performed by the enhanced chemiluminescence system (ECLTM PRN 2106; Amersham Pharmacia Biotech, Buckinghamshire, UK). The intensity of the bands was analysed with a gel documentation system (Bio-Rad Gel Doc 1000 and Multi-Analyst® version 1.1).

Quantitative real-time PCR

Renal tissues were harvested from mice 7 and 14 days after disease induction, and the cytokine expression profile was assayed with real-time PCR. Briefly, total RNA was isolated from renal tissues with the RNeasy kit (Qiagen GmBH, Hilden, Germany) and 1 µg of total RNA was reverse transcribed using oligo-d(T) primers and AMV-RT Taq polymerase (Promega). Real-time PCR was performed using Assay-on-Demand TaqMan probes and primers for TGF-β1, TGF-β1 and GAPDH (Applied Biosystems, Foster City, CA, USA) with an ABI PRISM 7500 Sequence Detection System. For blocking of the TGF β effect, Murine mesangial cells (1 × 10⁴/well, SV40 MES 13) were incubated with cysteamine (Sigma) for 1 h at 37°C before adding LPS (0.5, 1 and 2 µg/ml) in the in vitro system. In some experiments, cysteamine was used in different concentration (0.25, 0.5 and 1 mM). After 16 h, total RNA was isolated from mesangial cells. The transcript levels of TGF and TGF-β1 were analysed by real-time PCR. The results for each cytokine were normalized with respect to GAPDH expression.

Statistical analysis

The results are expressed as mean ± SD or mean ± SEM where appropriate. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). To compare more than two groups, the one-way ANOVA statistical analysis using the Tukey test was performed. P < 0.05 was used to indicate a statistically significant difference.

Results

Uteroglobin treatment attenuated the TGF expression and the severity of ecGN

To evaluate the renal cellular responses to exogenous stimuli, we quantified the degree of cell proliferation using a mouse mesangial cell culture system. Mesangial cells...
Fig. 1. (A) rUG has the direct suppressive capacity in the culture system. LPS stimulation (2 µg/ml) induced mesangial cellular proliferation. Mesangial cells (SV40 MES 13, 1 × 10^4/well) were added with/without rUG (25, 50 µg/ml) for 24 h. Cellular proliferation was measured by MTS absorbance. The rUG-treated Mesangial cells suppressed LPS-driven cellular proliferation. Each condition was triplicated and this represents one of three independent experiments (*P < 0.05). (B) rUG down-regulated the expressions of tTG and TGF-β1. Mesangial cells (SV40 MES 13, 1 × 10^4/well) were cultured with/without lipopolysaccharide (LPS, 2 µg/ml) and rUG (50 µg/ml). The transcript levels of tTG and TGF-β1 were analysed by real-time PCR after 24 h. The results for each cytokine were normalized with respect to GAPDH expression. Each condition was triplicated and this represents one of three independent experiments (*P < 0.05). (C) rUG lessened tTG expression. Mesangial cells (SV40 MES 13, 1 × 10^4/well) were obtained at 24 h after adding rUG. The culture media were changed with one containing 1% FBS, 1% FBS + LPS (2 µg/ml) and 1% FBS + rUG (10, 25 and 50 µg/ml). Deposition of tTG (green) was markedly inhibited with the rUG after the addition of LPS. DAPI (blue) was used as counter-staining (original magnification ×200).

proliferated with the addition of LPS but the cell proliferation was inhibited with the addition of rUG in a dose-dependent manner (Figure 1A). The cell proliferation was parallel with the up-regulation of tTG and TGF-β1. But the exogenous addition of rUG to culture media lessened the expression of tTG and TGF-β1 down to control levels (Figure 1B). Also, tTG expression of mesangial cells by LPS stimulation was inhibited by the exogenous rUG in a dose-dependent manner (Figure 1C).

To assess the relationship between tTG and UG in kidney disease, we induced ecGN using anti-GBM Ab. In a disease control group (anti-GBM Ab alone), cellular proliferation was evident in mesangial areas and fibrocellular crescents were developed in ~70% of glomeruli (73.3 ± 3.3%) at the 7th day and in 40% of glomeruli at the 14th day (46.7 ± 3.4%). Whereas with the treatment of rUG after the induction of ecGN, the histologic examination disclosed less crescent formation after 7 days and 14 days (30 ± 2.9%, 8.3 ± 1.7%, respectively; *P < 0.05 compared to disease control) (Figure 2A). Also, the GSI was higher in disease control mice compared to the mice treated with rUG after 2 weeks of disease induction, i.e. the majority...
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Fig. 2. (A) Presence of rUG attenuated crescent formation of GN. rUG treatment (0.5 mg/mouse, 0, +1 and +2 day) into B6 after anti-GBM Ab injection reduced the formation of cellular crescents and mesangial proliferation. Sections of 4 µm thick were stained with periodic acid Schiff's (PAS) reagent (original magnification ×200). Pictures were taken at 7 days and 14 days of disease. A minimum of 50 glomeruli per mouse kidney were evaluated, and the mean value was used as representative for the mouse (anti-GBM Ab versus anti-GBM Ab + rUG, **P < 0.01). (B) Glomerular sclerosis was lessened by the administration of rUG. Periodic acid Schiff (PAS) staining in mouse 7 days and 14 days after GN showing glomerulosclerosis (anti-GBM Ab versus anti-GBM Ab + rUG, **P < 0.01). Data are expressed as the mean ± SEM. (C) Effects of rUG treatment on histologic changes. Masson's trichrome-stained sections (original magnification ×200). In mice with ecGN, interstitial fibrosis and fibrin in glomeruli developed at Day 7 and Day 14 although no interstitial fibrosis was seen by the introduction of rUG at Day 7. Mean scores from three animals per group were then averaged (anti-GBM Ab versus anti-GBM Ab + rUG, **P < 0.05, **P < 0.01). (D) rUG administration has the antiproteinuric effect on ecGN. With the introduction of anti-GBM Ab, proteinuria as well as the deterioration of renal function developed in B6 mice, and the administration of rUG reduced the severity of ecGN. The results are expressed as mean ± SEM (n = 8/group, **P < 0.01, t test).

of glomerulus belongs to 3+ or 4+ grade of GSI in disease control mice while in rUG-treated mice, more than half of glomeruli showed 1+ or 2+ grade of the sclerosis index (Figure 2B). The interstitial and glomerular fibrosis were further confirmed by Masson's trichrome staining. As shown in Figure 2C, rUG treatment markedly attenuated the fibrotic process that was prominent in disease control mice. Overall, mice that were treated with rUG after anti-GBM Ab injection showed much mild histological changes. Also, the degree of renal impairment was parallel with the histological findings according to the treatment, i.e. the amount of proteinuria was significantly reduced in rUG-treated mice compared to that of disease control mice (108.0 ± 8 mg/mg creatinine versus 24.2 ± 5.8, P < 0.01) and the elevation of BUN was attenuated by rUG treatment (96 ± 9.6 mg/dl versus 45 ± 7.0, P < 0.01) at 7 days of disease (Figure 2D). These differences persisted until 2 weeks of disease (proteinuria 72.5 ± 8.0 mg/mg creatinine versus 22.5 ± 3.5, P < 0.01; BUN 56 ± 6.8 mg/dl versus 37 ± 4.5, P < 0.05).

Molecular changes according to treatment in ecGN

To probe the changes of molecular interactions in ecGN by the administration of rUG, we quantified the expressions of tTG and TGF-β1 in kidney tissues. TtG and TGF-β1 were up-regulated by the induction of ecGN, but they were lessened by rUG treatment as assessed by the protein expression (Figure 3A). The changes of protein expression were parallel with the transcriptional activities of these molecules as assessed by real-time PCR (Figure 3B). To delineate the spatial interactions of tTG and TGF-β1 in ecGN, we traced these proteins by confocal microscopy and immunohistochemistry. As shown in Figure 3C, tTG was expressed mainly on mesangial areas by the administration of anti-GBM Ab, and rUG treatment markedly
Fig. 3. (A) The administration of rUG into B6 mice suppressed tTG and TGF-β1 production. Kidneys were obtained from mice at Day 7 of ecGN. The protein levels of tTG and TGF-β1 were measured by western blot and blots were re-probed with anti-β-actin antibody as a loading control. The results are representative of three separate experiments (anti-GBM Ab versus anti-GBM Ab+rUG, **P < 0.01). (B) The transcript levels of tTG and TGF-β1 were analysed by real-time PCR after 7 and 14 days of disease induction. The results for each cytokine were normalized with respect to GAPDH expression. Each cytokine expression was differentially regulated according to the presence and the absence of rUG. Data are the mean ± SEM of three mice in each group (anti-GBM Ab versus anti-GBM Ab+rUG, *P < 0.05, **P < 0.01). (C) Glomerular expression of tTG was evident and rUG inhibited its expression. Renal tissues were harvested from mice 7 days after disease induction. The tTG expression was evaluated by confocal microscope analysis. The intraglomerular expression of tTG (red) was augmented after the induction of ecGN in B6 mice, but were markedly diminished in rUG-treated B6 mice. Each channel was separately scanned using a multitrack PMT configuration to avoid cross-talk between fluorescent labels. It was possible to alter the scan field size by a zoom facility, and further magnification up to 1000× allowed the detailed observations. The sections were counter-stained with DAPI (blue). (D) Effects of rUG on TGF-β1 expression of glomeruli. The intraglomerular expression of TGF-β1 was evident after the induction of ecGN in B6 mice at Day 7, but was markedly attenuated in rUG-treated mice (original magnification ×400). Data are the mean ± SEM of three mice in each group (**P < 0.01). (E) Intraglomerular expression of type IV collagen with the induction of ecGN. Type IV collagen was evident along Bowman's capsule with the induction of ecGN but was much lessened with rUG treatment (original magnification ×400). Sections were counterstained with haematoxylin. Type IV collagen positive cells were counted in 50 consecutive glomeruli or interstitial areas in randomized sections. Data are the mean ± SEM of three mice in each group (anti-GBM Ab versus anti-GBM Ab+rUG, *P < 0.05, **P < 0.01).
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Fig. 4. (A) Cysteamine blocked the tTG effect in the in vitro system. Mesangial cells (SV40 MES 13, 1 × 10^4/well) were stimulated by LPS (2 µg/ml) for 16 h. The culture media were added with/wtout cysteamine (0.25, 0.5 and 1 mM). The transcript levels of tTG and TGF-β1 were assessed by real-time PCR. The levels were normalized to those of GAPDH. Values are mean ± SEM of three different sample (compared to non-cysteamine *P < 0.05). (B) Mesangial cells (SV40 MES 13, 1 × 10^4/well) were stimulated by LPS (0.5, 1 and 2 µg/ml) for 16 h. The culture media were added with/wtout cysteamine (0.5 mM). The transcript levels of tTG and TGF-β1 were measured by real-time PCR. Real-time PCR assays were repeated in, at least, three doses, and basically similar results were obtained at each dose. The levels were normalized to those of GAPDH. Values are mean ± SEM of three different samples (compared to non-cysteamine. *P < 0.05, **P < 0.01).

attenuated the expressions of tTG in glomerular tufts without spatial changes. The expression of TGF-β1 appeared on the mesangial area along with tTG and was changed according to disease modification (Figure 3D). To probe the changes of glomeruli that were appeared to be normal by light microscopy, the deposition type IV collagen was traced by immunohistochemistry. In disease control mice, type IV collagen was frequently found in the parietal epithelial cells, but rUG treatment attenuated the deposition of fibrotic proteins along with the glomerular parietal epithelial cells (Figure 3E).

The tTG inhibition attenuated the harmful responses of renal cells
To evaluate the notion that the regulation of tTG expression would directly be associated with the disease severity,
we used cysteamine to block the tTG effect in the in vitro system. With the addition of LPS to mesangial cell culture, the expressions of tTG and TGF-β1 were up-regulated, whereas the addition of cysteamine along with LPS attenuated the expression of these cytokines, especially of tTG (Figure 4A). In a different concentration of LPS, the protective effect of TG2 inhibition was evident in terms of the expressions of TGF-β1 and tTG (Figure 4B).

Discussion

In this study, we evaluated the renoprotective effect of UG in ecGN. We showed UG attenuated the renal inflammation induced by anti-GBM Ab by modulating the expression of tTG and TGF-β1. Furthermore, we demonstrated that the inhibition of tTG by the specific inhibitor might lessen the severity of inflammatory responses in renal cells against noxious stimuli. ecGN was characterized by mesangial proliferation and matrix alteration with crescent formation [3,5,19]. Because the mechanism of development of ecGN is complicated and multiple factors are known to be involved, it is not easy to understand the role of one factor without the consideration of complex interactions with other variables. TGF-β1 and tTG are one of the examples; it is extensively studied that they are involved and play a critical role in various nephropathy, but the interaction of these two factors has not been thoroughly evaluated. In this study, we discovered the close relationship between tTG and TGF-β1 in the development of ecGN and the modulation by rUG led to the improvement of renal function and histopathologic changes.

TGF-β1 has been identified as an important proinflammatory and fibrogenic mediator to play a key role in mesangial cell apoptosis, accumulation of extracellular matrix and fibrosis [20]. TGF-β1 expression is essential in the progression of kidney injuries, i.e. after the induction of ecGN, TGF-β1 increased in urine and renal tissue, followed by remarkable tissue remodelling response. Recently, blockage of the TGF-β1-mediated signalling has been applied as a new renoprotective tool in the management of renal diseases [10,21].

tTG activity and its by-products were involved in various GN and correlated well with renal scarring [22]. Johnson et al. demonstrated the close relationship between tTG and renal dysfunction and histologic changes in human renal biopsy samples including 11 different glomerulonephritides [14]. Also, it is reported that tTG expression was associated with several clinical parameters such as serum creatinine, creatinine clearance and urinary protein excretion in IgA nephropathy [23]. In the studies utilizing human kidney samples, tTG expression was evident in mesangial area and proximal tubules [14,23]. In our study, tTG was evidently expressed in mesangial areas as well. In rat model of focal segmental glomerulosclerosis, tTG was observed within glomeruli showing capillary and mesangial distribution [24]. They also claimed that tubulointerstitial expression of tTG was hardly found. We further confirmed the localization of tTG expression using immunohistochemistry, which revealed similar findings with confocal microscopy (supplement figure). The relationship between TGF-β1 and tTG has been investigated by some researchers [13,25], but it was not evaluated thoroughly, especially in GN. In this study, we have demonstrated that tTG and TGF-β1 were up-regulated remarkably in the glomeruli after the induction of ecGN and these expressions were parallel with the deterioration of renal function as well as with histologic changes. We also demonstrated that rUG treatment lessened the severity of clinical illness in terms of renal function and histologic changes. Previously we have shown that the protective effect of rUG was exerted by preventing the deposition of harmful Ab into the site of interest [5]. In this study we suggest that the protective effect of UG might be derived from the modulation of the biologic effect of tTG as well. UG was first discovered more than three decades ago, and the biochemical properties and physiological functions have been considerably studied and established. According to the earlier studies, UG was considered as a substrate of TG 2, i.e. tTG catalyzed covalent links of UG to modulate the immunologic reaction [18]. Moreno et al., however, reported that anti-flamin, which was synthesized with the highest similarity with UG, inhibited tTG expression [26] and presented the possible role of UG as a tTG inhibitor. In ecGN, the preventive effect of UG was reported [5], but the inhibitory effect on the production of tTG and TGF-β1 was not demonstrated in the progression of renal injury. In this study, we hypothesized and confirmed that UG had an influence on tTG expression and TGF-β1 production. This blocking action of rUG led to a prominent improvement of glomerular injury accompanied by better preservation of renal function.

There are several limitations in this study that should be considered. First of all, the final end-point by controlling the in vivo tTG activity was not measured. Moreover, two isoforms of tTG were demonstrated showing that the full-length tTG mediated protective role against cellular insults and the shorter form promoted cell apoptosis [27]. Second, the cause–result relationship between the expressions of TGF and TGF-β1 was not directly evaluated in this study. Although we observed the spatial differences of expression of these two molecules using confocal microscopy, the discrepancy of expression sites should be considered for interpretation.

In conclusion, we demonstrate for the first time that rUG is able to attenuate renal injury via the modulation of expressions of tTG and TGF-β1 in ecGN, and suggest the possible molecular targets to reduce the severity of human GN.

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Conflict of interest statement. None declared.

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